Foxp3\(^+\) regulatory T cells promiscuously accept thymic signals critical for their development

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Foxp3\(^+\) regulatory T cells develop in the thymus and are essential for maintaining peripheral tolerance to self tissues. We report the critical requirement for CD154 up-regulation specifically on, and during the thymic development of, Foxp3\(^+\) regulatory T cells for the induction of their clonal expansion within the medulla. In the absence of this signal, there was a severe reduction in their thymic generation and output, leading to decreased peripheral numbers. Importantly, CD40 expression on either thymic dendritic or epithelial cells was sufficient to promote the development of normal numbers of Foxp3\(^+\) regulatory T cells. This work suggests that CD154-transduced signals promote Foxp3\(^+\) regulatory T cell development and highlights the plasticity of the thymic stroma for supporting their generation. Crucially, this study demonstrates that Foxp3\(^+\) regulatory T cells can promiscuously accept a single critical signal necessary for their thymic development from different cellular sources, redefining our understanding of their generation.

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egulatory T cells (Tregs) actively suppress autoreactive T cells, thereby limiting aberrant immune responses and maintaining tolerance toward self tissues (1). Deficiencies in this T cell subset in humans and mice can lead to severe autoimmunity. Initially, these suppressor cells were identified as CD4\(^+\) CD25\(^+\) T cells (2), a phenotype that also included activated CD4\(^+\) T cells, but, recently, Foxp3 has been characterized as the master regulator of their development and function (3–5) and is used as a highly specific marker for Tregs (6). Foxp3\(^+\) Tregs develop in the thymus as part of the normal T cell ontogeny processes (7), and Foxp3 expression is largely restricted to the thymic medulla and CD4\(^+\)CD8\(^-\) (CD4SP) thymocyte subset; the end stage of CD4\(^+\) T cell development. Thymic output of Foxp3\(^+\) Tregs is temporally delayed (8), and their development requires a high-affinity self-reactive T cell receptor (9, 10), the costimulatory molecule CD28 (11), and possibly IL-2 (12). It has been suggested that medullary thymic epithelial cells (mTECs), which express and present tissue-specific antigens (TSAs) on MHC class II (MHC II) molecules, drive Foxp3\(^+\) Treg selection (13). Similarly, dendritic cells (DCs) are thought to induce CD4\(^+\)CD25\(^+\) Tregs in the human thymus through a T cell antigen receptor (TCR)–peptide–MHC II interaction (14). However, the relative contributions of thymic dendritic and epithelial cells in promoting and supporting the development of a polyclonal Foxp3\(^+\) Treg repertoire are not clear. In addition, the cellular origin and temporal importance of the signals necessary for Foxp3\(^+\) Treg development have not been determined, and, therefore, the rules governing how developing Foxp3\(^+\) Tregs receive and process these signals are poorly understood.

We report that the CD40–CD154 pathway is critical for promoting the thymic development of Foxp3\(^+\) Tregs. CD154 up-regulation specifically on developing Foxp3\(^+\) Tregs promoted their clonal expansion within the thymic medulla, suggesting a critical role for CD154-transduced signals in their generation. However, CD40 expression on either thymic dendritic or epithelial cells was sufficient to promote the development of normal numbers of Foxp3\(^+\) Tregs, highlighting the plasticity of the thymic stroma for supporting their development. This demonstrates that Foxp3\(^+\) Tregs can promiscuously accept a single critical signal necessary for their development from either the thymic dendritic or epithelial cell compartments. Crucially, this implicates the potential uncoupling of all or some of the signals necessary for thymic Foxp3\(^+\) Treg generation, which has major implications for modulating their development for the potential therapy of autoimmune disease.

### Results

**CD40-Expressing Thymic Dendritic or Epithelial Cells Can Promote Foxp3\(^+\) Treg Development.** The interaction of CD40, expressed on antigen-presenting cells (APCs), and CD154, expressed on activated CD4\(^+\) T cells, provides costimulation for T cell priming and for the generation of thymus-dependent humoral immune responses (15). In mice deficient in this pathway, there is an absence of Ig class switching and germinal center formation. More recently, a severe reduction in the CD4\(^+\)CD25\(^+\) Treg population also has been observed (16–19). To assess the role of CD40–CD154 in Foxp3\(^+\) Treg development and homeostasis, we investigated Foxp3\(^+\)CD4\(^+\) T cells in WT, CD40\(^-/-\), and CD154\(^-/-\) mice. In contrast to WT mice both CD40\(^-/-\) and CD154\(^-/-\) mice had a reduction of between 50% and 60% Foxp3\(^+\) Tregs in both the thymus and the periphery (Fig. 1A). This demonstrates a critical role for CD40–CD154 in the thymic development of Foxp3\(^+\) Tregs and may suggest a secondary role in their peripheral homeostasis.

CD40 is expressed on thymic DCs and has been observed by immunoelectron and immunofluorescent microscopy on cortical thymic epithelial cells (cTECs) and some mTECs (20, 21). We resolved to define the thymic expression of CD40 by flow cytometry. In WT mice, CD40 was expressed on freshly isolated conventional CD11c\(^+\) DCs (cDCs), cTECs, and mTECs (Fig. 1B–D; see supporting information (SI) Fig. 5 A and B for additional information on the identification of cTECs and mTECs). As mTECs mature, they up-regulate CD80 and Aire, acquiring the capacity to express and present TSAs (22, 23). We found that CD80\(^+\) mTECs expressed higher levels of CD40 than CD80\(^-\) mTECs (Fig. 1D), correlating CD40 expression with self-antigen presentation. Although functional CD40 expression has been reported on thymocytes and peripheral T cells (24, 25), we found no expression of CD40 on activated CD4\(^+\) T cells or CD4SP thymocytes (SI Fig. 5 C and D). Thus, CD40 is expressed under steady-state conditions at high levels on the majority of thymic DCs, cortical thymic epithelial cells, and CD80\(^+\) medullary thymic epithelial cells.

To determine the cellular source of CD40 that promoted Foxp3\(^+\) Treg development, we generated bone marrow chimeric...
mice in which the recipient immune system was ablated with irradiation and reconstituted with donor bone marrow. We produced mice expressing CD40 on combinations of radiosensitive thymic epithelial cells and radiosensitive hematopoietic DCs (Fig. 1 E and SI Fig. 5 E and F). Strikingly, CD40 expression on either thymic dendritic or epithelial cells was sufficient to promote the development of normal numbers of Foxp3+ Tregs (Fig. 1 F), expressing comparable levels of Foxp3 to those that developed in the presence of CD40 on both thymic dendritic and epithelial cells (data not shown). This is direct evidence that both thymic dendritic and epithelial cells have the capacity to promote the generation of a polyclonal Foxp3+ Treg population.

**CD40–CD154 Promotes Foxp3+ Treg Development Independently of CD80/CD86–CD28.** Ligation of CD28 on T cells by APC-expressed CD80/CD86 is critical not only for T cell activation but also for the thymic development and peripheral homeostasis of Foxp3+ Tregs (11). It has been suggested that CD40 ligation is required for the up-regulation and maintenance of high-level MHC II, CD80, and CD86 expression on peripheral DCs (26) and for normal thymic CD86 expression (27). Additionally, RANK, a member of the TNF receptor superfamily that shares sequence homology with CD40 (28), regulates the maturation, and therefore antigen-presenting capacity, of mTECs (23). We examined whether thymic CD40 expression promoted Foxp3+ Treg development through the manipulation of CD80/86 or the antigen-presenting capacity of the thymic stroma. The maturation of mTECs, as measured by CD80 expression, was not altered in CD40−/− mice, compared with WT, mice (Fig. 2 A). Likewise, the expression of CD80, CD86, and the MHC II molecule I-Aβ was comparable on freshly isolated WT and CD40−/− thymic cDCs, cTECs, and mTECs (although thymic epithelial cells expressed very little or no CD86) (Fig. 2 B and D). This was also true for resting peripheral cDCs (SI Fig. 6). This clearly suggests that CD40 does not regulate thymic antigen presentation or costimulation through CD80/CD86. Furthermore, blockade of CD80/CD86 in CD154−/− mice led to a second severe reduction in thymic and peripheral Foxp3+ Tregs (Fig. 2 E), indicating that although both the CD80/CD86–CD28 and CD40–CD154 pathways are critical for Foxp3+ Treg development, their requirement is independent of one another.

**CD154 Is Required Specifically in Developing Foxp3+ Tregs.** Whereas activation of CD4+ T cells by the CD40–CD154 pathway is regulated indirectly through the CD40-mediated activation and maturation of DCs, a role for CD154 signaling in costimulation has also been recognized (29, 30). Because CD40 did not appear to regulate other known mediators of Foxp3+ Treg development, we considered the role of CD154 in their generation. Thymic CD154 expression is reported to localize in the medulla and increase after TCR stimulation (20). Activated peripheral CD4+ T cells are also reported to up-regulate CD154 (16). In the thymus of WT mice, we found that activation induced CD154 up-regulation on most Foxp3+ CD4SP thymocytes but only on minor populations of Foxp3− CD4SP and the less mature CD4+CD8+ (DP) thymocytes (Fig. 3 A). The capacity of Foxp3+ Tregs to up-regulate CD154 was further reduced in the periphery (SI Fig. 7 A–C). However, CD154 expression was markedly elevated on resting and activated CD4+ T cells from the periphery and, to a lesser extent, the thymus of CD40−/− mice. This may suggest that CD40 suppresses CD154 expression and may thus negatively regulate T cell activation and, potentially, Foxp3+ Treg development (SI Fig. 7 D and E).
The CD40–CD154 pathway is crucial for negative selection of autoreactive thymocytes (31, 32). However, WT and CD154−/− thymocytes undergo equivalent and efficient negative selection when they coexist in the thymus of mixed bone marrow chimeric mice, indicating that CD154 signaling is not required specifically on those cells undergoing negative selection (32). We adopted a similar approach to determine whether CD154 expression is required specifically on those thymocytes that commit to the Foxp3+ Treg lineage, utilizing the congenic marker Thy1 to differentiate between Thy1.1+ WT and Thy1.2+ CD154−/− thymocytes. Whereas Foxp3+ Treg development was severely reduced in irradiated WT mice reconstituted with CD154−/−, rather than WT, bone marrow (Fig. 3 B and C), Foxp3 was preferentially up-regulated in WT, rather than CD154−/− thymocytes when they coexisted in a single thymus (Fig. 3 D and E). This clearly argues that promotion of Foxp3+ Treg development through the CD40–CD154 pathway depends on the up-regulation of CD154 specifically on developing Foxp3+ Tregs. CD154 signals are now strongly indicated as key mediators of Foxp3+ Treg development, and the relative paucity of CD154 expressed by Foxp3+ CD4SP thymocytes (Fig. 3A) indicates that CD154 may function before Foxp3 induction.

**CD154 Induces the Clonal Expansion of Foxp3+ Tregs.** Costimulation by CD40, expressed on thymic epithelial cells, specifically induces the clonal expansion of human CD4SP thymocytes in vitro (33). To assess whether CD154 expression regulated Foxp3+ Treg development in this way, we directly measured Foxp3+ Treg turnover in vivo by assessing BrdU incorporation in the DNA of proliferating cells 24 h after injection. In peripheral tissues, BrdU incorporation in Foxp3+ CD4+ T cells was reduced in the absence of CD154, indicating a reduced capacity to proliferate, yet the turnover of Foxp3+ CD4+ T cells was comparable in WT and CD154−/− mice (Fig. 4 A and B). In the thymus, turnover of the immature subsets of developing T cells, CD4+ CD8− (DN) and DP thymocytes, was comparable between WT and CD154−/− mice (Fig. 4C). However, CD154−/− Foxp3+ CD4SP and Foxp3− CD4SP thymocytes had considerably reduced BrdU incorporation, demonstrating dramatically reduced clonal expansion (Fig. 4C). Therefore, although thymocytes do not require CD154 for the rapid proliferation that they undergo during the DN and DP phases of T cell development, their turnover does require CD154 expression once they mature into CD4SP thymocytes and, subsequently, up-regulate Foxp3. Thus, Foxp3+ Treg development was abrogated in mice lacking CD154, at least in part, by an inability to expand the population of Foxp3+ CD4SP thymocytes that reside in the medulla. However, whereas peripheral CD154−/− nonregulatory CD4+ T cells also had a deficit in their capacity to proliferate, Foxp3+ CD4+ T cells did not, a finding that challenges the current belief that the observed decrease in Foxp3+ Tregs in the periphery of CD40−/−
and CD154−/− mice is the result of reduced homeostasis (16); instead, the present findings demonstrate that the cause is reduced thymic output.

**Discussion**

In summary, up-regulating CD154 during Foxp3+ Treg development ensures the delivery of critical signals required for promoting their generation. The necessity for CD154 expression specifically on those thymocytes that commit to the Foxp3+ Treg lineage, and the promiscuous acceptance of CD40 expression on either thymic dendritic or epithelial cells, suggests that CD154-transduced signals directly promote Foxp3+ Treg development. That CD154 is expressed at only minor levels on Foxp3+CD4SP thymocytes suggests that its critical role in Foxp3+ Treg development lies before Foxp3 induction. However, whether CD154 expression can directly induce Foxp3 is not known. Likewise, the role of CD154 expression in imprinting suppressor function on Foxp3+ Tregs during their development remains to be determined. Yet, it is clear that CD154 expression promotes Foxp3+ Treg development, at least in part, by inducing clonal expansion of the Foxp3+ CD4SP thymocyte pool within the medulla, a proliferative response that may be temporally distinct from an earlier CD154 signal. After this phase of expansion, Foxp3+ Tregs leave the thymus and populate the periphery, losing the capacity to up-regulate CD154, which then plays no further role in their homeostasis.

This work furthers our understanding of the signaling pathways that control Foxp3+ Treg development. Self-antigen presentation by mTECs drives the selection of Foxp3+ Tregs (13), and we show that CD40 and CD80, expressed on thymic DCs and epithelial cells, and CD86, expressed on thymic DCs, provide critical signals that promote their generation. More specifically, we identified that CD154-transduced signals may play a critical role in promoting thymic Foxp3+ Treg generation by inducing their clonal expansion, providing an appropriate therapeutic target that could potentially be exploited to specifically modulate thymic Foxp3+ Treg development.

However, in addition to identifying a specific pathway critical for Foxp3+ Treg development, this work redefines our general understanding of the generation of Foxp3+ Tregs by clearly demonstrating the plasticity of the thymic stroma for promoting Foxp3+ Treg development. Medullary thymic epithelial cells (13) and thymic DCs (14) have been implicated in directing Foxp3+ Treg development in mice and humans, respectively, yet their relative contributions in a single system remain to be defined. We demonstrate that both thymic dendritic and epithelial cells can promote the development of a polyclonal Foxp3+ Treg population and that they can do so through a common pathway. We therefore establish that developing Foxp3+ Tregs can promiscuously accept a single critical signal necessary for their development from different cellular sources. This implicates the potential uncoupling, in space and time, of all or some of the critical signals necessary for thymic Foxp3+ Treg
generation, demonstrating an inherent flexibility, or promiscuity, in Foxp3+ Treg lineage-committed thymocytes for receiving and processing signals that promote their generation. Crucially, this promiscuity makes developing Foxp3+ Tregs more susceptible to manipulation, and this should be exploited when modulating their development for the potential therapy of autoimmune disease.

Materials and Methods

Animals. All animals were housed under specific pathogen-free conditions in accordance with University of Cambridge regulations. Experimental procedures were performed on male and female mice, 5 and 7 weeks old, according to United Kingdom Home Office regulations.

Isolation of Thymic Stromal Cells. Isolated thymi were washed, trimmed of connective tissue, and finely minced. The majority of free thymocytes was removed by incubation at 37°C for 10 min in RPMI medium 1640. The supernatant was removed, and the remaining tissue fragments were digested with three incubations at 37°C for 30 min in RPMI medium 1640 containing 0.5 mg/ml collagenase, 0.3 mg/ml Dispase, and 8 μg/ml DNase I. The supernatants were pooled, and thymic rosettes were disrupted with incubation at 4°C for 15 min in PBS containing 5 mM EDTA. The cells were filtered and enriched for connective tissue, and finely minced. The majority of free thymocytes was removed by incubation at 37°C for 10 min in RPMI medium 1640 containing 0.5 mg/ml collagenase, 0.3 mg/ml Dispase, and 8 μg/ml DNase I. The supernatants were pooled, and thymic rosettes were disrupted with incubation at 4°C for 15 min in PBS containing 5 mM EDTA. The cells were filtered and enriched for thymic stromal cells on a discontinuous Percoll gradient as follows: cells were retrieved from the upper interface.

In Vitro Stimulation. For T cell and thymocyte activation, 10⁷ freshly isolated splenocytes or thymocytes were incubated for 3 h at 37°C in complete RPMI medium 1640 containing 12 mg/ml collagenase, 0.3 mg/ml Dispase, and 8 μg/ml DNase I. The supernatants were removed, and the remaining tissue fragments were digested with incubation at 4°C for 15 min in PBS containing 5 mM EDTA. The cells were filtered and enriched for thymic stromal cells on a discontinuous Percoll gradient as follows: cells were resuspended in Percoll (ρ, 1.115), overlaid with an equal volume of Percoll (ρ, 1.050) and then PBS, and centrifuged at 1,350 × g for 30 min. Thymic stromal cells were retrieved from the upper interface.

In Vivo Studies. For CD80/CD86 blockade, mice were injected i.p. with 100 μg each of purified anti-CD80 (16–10A1) and anti-CD86 (GL-1) (Bio Express) or appropriate isotype controls every second day for 14 days. Mice were analyzed 24 h after the final injection. For proliferation studies, mice were injected i.p. with 2 mg of BrdU and analyzed 24 h later.

Bone Marrow Chimeric Mice.Recipient female mice, 6 weeks of age, were lethally irradiated with two doses, given 3 h apart, of 600 rad from a caesium source. Bone marrow was prepared from the femurs and tibias of donor female mice, 8 weeks of age, and T cells were depleted by incubating with CD4 and CD8 microbeads and purifying on MACS Separation LD columns (Miltenyi Biotech). Recipient mice were injected i.v. with 10⁷ T cell-depleted bone marrow cells and maintained on 0.25% oral Baytir for 14 days. Bone marrow chimeric mice were analyzed 8–10 weeks later.

Antibodies and Flow Cytometry. CD4 (14–5211) FITC, CD4 (RM4–5) PerCP-Cy5.5 or Pacific Blue, CD8 (53–6–7) Pacific Blue, CD40 (3/23) FITC or phycoerythrin (PE), CD45 (30–F11) PerCP-Cy5.5, biotinylated CD80 (16–10A1), CD86 (GL1) FITC, CD154 (MR1) PE, purified epithelial cellular adhesion molecule (EpCAM) (G8.8), I-Ab (AF6–120.1) FITC, Ly-51 (BP-1) PE, and streptavidin APC, and Thy1.2 (53–2.1) FITC were purchased from eBioscience. EpCAM Pacific Blue was generated by using the Pacific Blue monoclonal antibody labeling kit (Invitrogen). Intracellular Foxp3 (FKJ-16s; eBioscience) and BrdU incorporation (BrdU FITC flow kit; BD Biosciences) were detected according to the manufacturer’s instructions. Flow cytometry was performed on a CyAn ADP (Dako) and analyzed by using FlowJo software (Tree Star).

Statistical Analysis. Statistical significance between two groups was assessed by using the nonparametric Mann–Whitney test. A P value of <0.05 was considered to be statistically significant.

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A. Foxp3-CD4+

B. Foxp3-CD4+

C. Foxp3+CD4+

D. CD154

E. CD154

% of Max

SSC

hIgG

resting

activated

CD154

resting

activated

CD154